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URAD RS ZA INTELEKTUALNO LASTNINO
REPUBLIKA SLOVENIJA

LEK, tovarna farmacevtskih in kemičnih izdelkov d.d.

Nov in izboljšan fermentativni postopek pridobivanja klavulanske kisline in njenih soli

Področje tehnike, v katero spada izum
(MPK C 12 P 17/18)

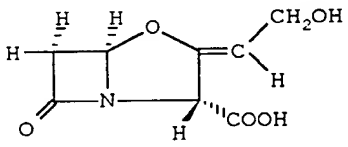
Predloženi izum spada v področje farmacevtske industrije in se nanaša na nov in izboljšan postopek za pripravo klavulanske kisline in njenih soli s fermentacijo mikroorganizma Streptomyces sp. P 6621 FERM P 2804.

Tehnični problem

Obstaja stalna potreba po novem in izboljšanem mikrobiološkem postopku za pripravo klavulanske kisline in nadalnje predelave v njene alkalijske soli, kot kalijevega klavulanata, po katerem bi želeno spojino dobili v visokem dobitku v vodnih gojilnih juhah, ki jih dobimo po fermentaciji s klavulansko kislino proizvajajočim mikroorganizmom.

Stanje tehnike

Klavulanska kislina je generični naziv za (2R,5R,Z)-3-(2-hidroksietiliden)-7-okso-4-oksa-1-azabiciklo/3.2.0/ heptan-2- karboksilno kislino s sledečo formulo:



Njene alkalijske soli in estri delujejo kot inhibitorji ß-laktamaz, ki jih proizvajajo nekateri gram pozitivni in gram negativni mikroorganizmi. Klavulanska kislina in njene alkalijske soli imajo poleg aktivnosti inhibicije ß-laktamaz še sinergistični učinek v kombinaciji z ß-laktamskimi antibiotiki penicilinske in cefalosporinske vrste, zato se klavulanska kislina in njene soli uporabljajo v galenskih pripravkih, da preprečijo deaktivacijo ß-laktamskih antibiotikov. Komercialni preparati vsebujejo bolj stabilno kalijevo sol klavulanske kisline (sama kislina je precej nestabilna) v kombinaciji z amoksicilin trihidratom, njegovimi solmi ali v kombinaciji z drugimi antibiotiki.

Klavulansko kislino pripravijo s fermentacijo mikroorganizma, ki proizvaja klavulansko kislino, kot so razni mikroorganizmi, ki pripadajo raznim sevom Streptomyces, kot so S.clavuligerus NRRL 3585, S.jumoninensis NRRL 5741, S.katsurahamanus IFO 13716 in Streptomyces sp. P 6621 FERM P 2804.

Uporaba S.clavuligerus NRRL 3585 v procesu priprave klavulanske kisline s fermentacijo je opisana v patentu GB 1 508 977, kjer je kot najbolj primerno za fermentacijo opisano gojišče, ki vsebuje med 0.1% in 10% organskega vira dušika, kot na primer hidrolizat kvasovk, mesni in ribji ekstrakt, semenski proteini, koruzni sirup in različni hidrolizati (v primeru definiranega gojišča se kot vir dušika uporabijo urea, valin, asparagin, glutaminska kislina, prolin in fenilalanin), med 0.1% in 5% organskega ogljika (hidrolizat škroba, dekstrin, saharoza, laktoza, glicerol in njegovi estri, rastlinska olja ter živalske maščobe). Kot posebej primeren in poceni medij se je pokazala kombinacija sojine moke, posušenega filtrata iz proizvodnje piva in dekstrina. Kemijsko definiranim gojiščem pa lahko dodamo še anorganske soli: natrijeve, kalijeve, magnezijeve, železove in cinkove kloride ter natrijeve, magnezijeve in železove sulfate ter natrijeve in kalijeve soli fosforne kisline. V gojišče se lahko dodajo tudi soli mangana, niklja in kobalta ter vitamini, za kontrolo penjenja pa je potrebno dodajanje antipenilcev. Rezultat fermentacije je fermentacijska brozga bogata s klavulansko kislino in njenimi solmi.

Po fermentaciji nastalo vodno gojilno juho lahko očistijo in koncentrirajo po običajnih postopkih, ki obsegajo npr. filtracijo in kromatografska čiščenja, kot je ponazorjeno v patentu GB 1 508 977, ki opisuje fermentacijski postopek pridobivanja klavulanske kisline z mikroorganizmom Streptomyces clavuligerus in njeno izolacijo iz kulture filtrata. Med drugim pa prikazuje tudi, da se soli klavulanske kisline lahko dobi z adsorpcijo klavulanatnega aniona iz filtrirane juhe na anionsko izmenjevalno smolo, pri čemer se iz nje eluirajo klavulanatni anion z elektrolitom, nastalo raztopino razsolijo in zatem odstranijo topilo. S tem postopkom dobijo sprejemljive dobitke želene snovi, vendar pa postopek temelji na zahtevnem čiščenju s kromatografskimi metodami, kot je znano, pa uporaba smolnih kolon terja znatne investicije, kar omejuje uporabo v velikem merilu.

Veliko novost in izboljšavo, ki omogoča uporabo v velikem merilu, predstavlja v našem slovenskem patentu P-9400107 oziroma ekvivalentni PCT prijavi WO 95/23870 opisana metoda izolacije klavulanske kisline oz. njene farmacevtsko sprejemljive alkalijske soli, kot kalijevega klavulanata, katere osnova je odstranitev micelija in drugih suspendiranih delcev iz fermentacijske juhe z mikrofiltracijo, v nato pa še z ultrafiltracijo. Tako prečiščeno juho nato koncentriramo z reverzno ozmozo in jo lahko direktno

ekstrahiramo z vodo nemešljivim organskim topilom, kar da po končanem postopku izolacije željeno spojino visoke čistote. S to metodo se izognemo zamudnim konvencionalnim metodam izolacije in kromatografskemu čiščenju produkta.

Iz literarature je znano, da je za čimbolj uspešno fermentacijo potrebno optimizirati pogoje v fermentorju. Članek Yung T.B. et al., Ferm.Technol.1972, Today 163 opisuje šaržno fermentacijo kot dinamičnen proces, pri katerem se okolje stalno spreminja, tako da so mikrobne celice stalno podvržene spremembam. Spremembe v okolju celice se izražajo kot znatne spremembe v celičnem fiziološkem stanju, tako da celice živijo stalno pod nekimi stresnimi dejavniki, ki onemogočajo njihovo optimalno rast in proizvodnjo želenih metabolitov v njih.

Tako na primer patent GB 1 543 563 opisuje modificiran fermentativni postopek pridobivanja klavulanske kisline z uporabo seva S.clavuligerus NRRL 3585, pri katerem vzdržujejo vrednost pH medija v območju med 6.3 in 6.7, s čemer zvišajo dobitek želene spojine.

Poleg fizikalnih parametrov kot pH, temperatura, vsebnost kisika, lahko v fermentorju kontroliramo in vzdržujemo tudi količino asimilirnih virov dušika in ogljika ter količino raznih virov mineralov. Tako vodeno fermentacijo imenujemo dohranjevalna fermentacija, ki je dobro poznana tudi iz literature. Uporaba takšne fermentacije je opisana v knjigi Crueger W. et. al., BIOTEHNOLOGY 1984, p. 197-237., kjer je med drugim opisana uporabnost dohranjevalne fermentacije tudi v proizvodnji antibiotikov.

Primer kontroliranja in vzdrževanja količine asimilirnega vira dušika in ogljika v fermentacijski brozgi je opisan v članku LEE J.S. et. al., Kor.Jour.Microbiol. 1978, Vol 15, No. 1, p. 21-29., ki opisuje, da proizvodnjo penicilina lahko izboljšamo s kontroliranim dodajanjem asimilirnega vira dušika in ogljika, tako da stalno sledimo potrebam mikroorganizmov v fermentorju.

V članku Lilley G. et. al., J.Chem.Tech.Biotehnol. 1981, Vol. 31, p. 127-134. je opisano, da proizvodnjo nekaterih antibiotikov z bakterijami vrste Streptomyces lahko reguliramo z ustreznim spreminjanjem koncentracije asimilirnega vira dušika, ogljika in vira fosforja. Tako se na primer

proizvodnja thienamycina v fermentorju prične šele, ko količina fosforja pade proti ničli.

Uporaba dohranjevanja in kontrole količine asimilirnega vira ogljika v procesu proizvodnje klavulanske kisline je prvič opisana v EP-B-0 182 522, ki opisuje metodo priprave klavulanske kisline s šaržno fermentacijo mikroorganizma S.clavuligerus, pri čemer so ugotovili pomembno izboljšanje postopka, v kolikor vir ogljika, kot glicerol, dodajamo v gojišče med fermentacijo bodisi kontinuirno, bodisi v presledkih, pri čemer je zelo pomembno, da vzdržujemo nivo ogljika pri dovolj nizki koncentraciji in sicer pod 0,5% (w/v), nikakor pa ne sme nivo ogljika narasti preko 2%. Primeri ponazarjajo, da je bistvena izboljšava v zvišanem dobitku klavulanske kisline opažena, ko vir ogljika, kot glicerol, v izhodnem hranljivem gojišču ni bil prisoten in se je dodajal med fermentacijo kontinuirno ali v presledkih. Navajajo, da je koncentracija klavulanske kisline v fermentacijski juhi po 160 urah znašala okoli 1400 μg/ml, kar je opazno izboljšanje glede na prejšnje postopke.

Iz članka Butterworth D., Drugs Pharm.Sci.1984, Vol 22 (Biotehnol. Ind. Antibiot.) p. 225-35., ki prikazuje pregled objavljenih fermentacijskih postopkov pridobivanja klavulanske kisline z znanimi vrstami mikroorganizmov rodu Streptomyces, je razvidno, da postopki pridobivanja klavulanske kisline z mikroorganizmi S. clavuligerus, ki so objavljeni v patentni literaturi, temeljijo le na pilotnih napravah ter da so postopki pridobivanja klavulanske kisline z drugimi mikroorganizmi rodu Streptomyces omejeni le na manjše oziroma laboratorijsko merilo. Tudi fermentacijski postopek pridobivanja klavulanske kisline, ki je opisan v patentu EP-B-0 182 522, je prikazan le v pilotem merilu ("pilot plant scale procedures"), tako da dosedaj objavljena literatura ne opisuje fermentacije klavulanske kisline z mikroorganizmi rodu Streptomyces v industrijskem merilu ("large-scale operation").

Opis rešitve tehničnega problema s primeri

Izum temelji na nalogi bistveno zvišati dobitek oziroma koncentracijo klavulanske kisline v vodni gojilni juhi, pripravljeni s fermentacijo mikroorganizma Streptomyces sp. PP 6621 FERM P 2804.

Ta smoter smo dosegli na način, da se mikroorganizem Streptomyces sp. P 6621 FERM P 2804, ki je opisan v JP Kokkai Application No. 79-72 085 (Early-Disclosure No. 80-162993) in proizvaja klavulansko kislino, goji pri temperaturi med 20° do 30°C, najbolje pri temperaturi med 23° do 25°C in pri vrednosti pH medija med 6.5 do 7.5, najbolje pri kontroliranem vzdrževanju vrednosti pH medija med 6.8 do 6.9 z vodno raztopino alkalijskega sredstva, v aerobnih pogojih in hranljivem gojišču, ki vsebuje vir ogljika, vir dušika in mineralne soli.

Izraz gojenje pomeni prosto aerobno rast organizma, ki proizvaja klavulansko kislino v prisotnosti vira ogljika, vira dušika in mineralnih soli, ki se lahko asimilirajo. Takšna aerobna rast poteka v hranljivem gojišču, v katerem so raztopljene ali suspendirane hranljive sestavine. Gojenje lahko poteka na aerobni površini ali submerzno. Hranljivo gojišče je lahko sestavljeno iz kompleksnih hranljivih sestavin ali iz kemično definiranih sestavin.

Hranljive podloge, ki jih uporabljamo za gojenje Streptomyces sp. P 6621 FERM P 2804 so v množini od 2% do 15% kompleksni viri organskega dušika, kot so lahko ekstrakti kvasa, rib in mesa, rastlinski proteini, kot sojina moka, hidrolizati proteinov, kot peptoni. Izbirno se lahko uporabijo tudi kemično definirani viri dušika, kot so različne amino kisline.

Kot vir ogljika v množini od 1.5% do 7.5% za hranljivo gojišče lahko uporabimo katerikoli asimilirni vir ogljika, najbolje tisti s kemično definirano strukturo. Lahko uporabimo škrob ali škrobne hidrolizate, kot je dekstrin, saharoza, laktoza, maltoza in drugi sladkorji ali glicerin in glicerinske estre, kot glicerol trioleat (Estol). Kot vir ogljika lahko uporabimo še razna rastlinska olja, kot olje iz semen bombaža. Dodatek sredstev proti penjenju, kot je Synperonic (komercialni naziv firme I.C.I., GB), je nujen za kontrolo penjenja v gojišču fermentorja.

V zgoraj navedena gojišča se dodaja še mineralne soli, v množini okoli 0.05%, kot so $MgCl_2$, $FeCl_3$, $ZnCl_2$, $CuCl_2$, $MnSO_4$, $FeSO_4$, Na_2SO_4 in natrijeve ali kalijeve soli fosforjeve kisline, kot NaH_2PO_4.

V sledovih se lahko vključi še nekatere elemente, kot kobalt idr.

Streptomyces sp. P6621 FERM P 2804 se lahko goji v zgoraj opisanih gojiščih v steklenih erlenmajericah, kjer poteka aeracija s stresanjem na rotacijskih stresalnikih ali v konvencionalnih aerobnih fermentorjih iz nerjavečega jekla, opremljenih za mešanje in dovod sterilnega zraka. Delovni volumen fermentorja znaša od 25% do 85% celotne kapacitete, fermentacija pa lahko poteka šaržno ali kontinuirno.

Najvišje dobitke klavulanske kisline dobimo v času do največ 6 dni.

V skladu s smotrom izuma, kjer opisujemo šaržno fermentacijo Streptomyces sp. P 6621 FERM P2804 najprej s selekcijskim postopkom izoliramo najustreznejši klon mikroorganizma. Nato pripravimo laboratorijski inokulum, ki je visokoproduktivna kultura na poševnikih ali rujevkah.

Sledi fermentacija mikroorganizma, pri čemer za dosego najboljših rezultatov najprej nacepimo laboratorijski inokulum v propagator (predpredfermentor), s predhodno pripravljenim steriliziranim gojiščem. Gojišče na tej stopnji je rastno, vegetativno in je namenjeno le namnožitvi mikroorganizma in ne še pridobivanju klavulanske kisline. Ko kultura doseže želeno stopnjo rasti (18 do 25%-no razbarvanje v 0.5 do 1.5 min)(procent razbarvanja je merilo za vitalnost kulture), jo precepimo v predfermentor, s predhodno pripravljenim steriliziranim gojiščem. Tudi ta stopnja je vegetativna in je namenjena le namnožitvi mikroorganizma. Šele nato, ko kultura doseže želeno stopnjo rasti, jo nacepimo v sterilizirano gojišče fermentorja, kjer nato poteka mikrobiološki postopek pridobivanja klavulanske kisline.

Za sestavo gojišča uporabimo vir dušika, vir ogljika in mineralne soli, kot so zgoraj navedeni, postopki rasti in mikrobiološko pridobivanje klavulanske kisline pa je ponazorjeno v primeru.

Iz patenta EP-B-0 182 552 je znano, da delež ogljika v gojišču ob inokulaciji znaša med 0% do 2% (w/v)(weight/volume), najbolje od 0% do

0.5% (w/v). Ta pomen je ponazorjen tudi v primerih 1 do 3, kjer dobijo najboljše rezultate, če v začetnem gojišču vira ogljika sploh ni prisotnega. V skladu z opisanim primerom 1 so po končani fermentaciji po 160 urah dosegli visok dobitek oziroma koncentracijo klavulanske kisline, ki je znašala okoli 1400 μg/ml.

Za razliko od mikrobiološkega postopka pridobivanja klavulanske kisline s šaržno fermentacijo S.clavuligeris, kot je opisano v patentu EP-B-0 182 522, kjer so dosegli izrazito izboljšavo na način, da so v začetno gojišče, kjer vir ogljika ni bil prisoten, med fermentacijo dodajali kontinuirno ali v presledkih vir ogljika na način, da so koncentracijo ogljika vzdrževali pod 0.5% (w/v) ali najbolje še nižje, nikakor pa koncentracija vira ogljika ni smela v nobenem trenutku preseči 2%, smo sedaj presenetljivo ugotovili, da klavulanska kislina nastane v bistveno višjem dobitku oziroma v višjih koncentracijah v vodnih gojilnih juhah s fermentacijo mikroorganizma Streptomyces sp. P 6621 FERM P2804, kjer je v začetnem gojišču prisoten vir asimilirnega ogljika in dušika ter vir fosforja, tekom fermentacije pa dodajamo dodatne množine vira fosforja in vira asimilirnega dušika.

V skladu s smotrom izuma smo nadalje ugotovili, da neprisotnost vira ogljika v izhodnem gojišču, glede na navedbe iz EP-B-0 182 552, ni pomembna in da lahko uporabimo začetno gojišče, kjer delež ogljika (npr. glicerol, glicerol trioleat in koruzni škrob) znaša več kot 5% (w/v). Za doseganje kar največjih dobitkov klavulanske kisline je pomembno, da imamo v začetnem gojišču dovolj visoko koncentracijo fosforja, vsekakor pa višjo od 0.02% (w/v) in manjšo od 0.15% (w/v). V nadaljevanju fermentacije pa najdlje do štiridesete ure fermentacije ohranjamo zadostno koncentracijo fosforja med 0.15% in 0.005% (w/v) z dodajanjem vira fosforja, potem pa pustimo, da se koncentracija fosforja znižuje proti nič (w/v). S tem reguliramo rast biomase in preprečimo prezgodnjo proizvodnjo klavulanske kisline. (Ko se v procesu fermentacije začne proizvodnja sekundarnih metabolitov, kar klavulanska kislina vsekakor je, se proizvodnja žive biomase upočasni.)

V skladu s smotrom izuma smo nadalje ugotovili, da dodajanje amonijevega hidroksida v fermentacijsko brozgo od začetka do konca fermentacije kot edinega asimilirnega vira dušika in istočasno tudi regulatorja pH ne vpliva pozitivno na potek fermentacije. Prevelika količina amoniaka pomeni strup za

celice, prenizek pH pa manjšo proizvodnjo klavulanske kisline, zato najprej dodamo v fermentacijsko brozgo kot asimilirni vir dušika sojino moko, nato pa dodajamo amonijev hidroksid.

V primeru, da smo kot asimilirni vir dušika namesto amonijevega hidroksida uporabili amonijev sulfat, kot regulator pH pa natrijev hidroksid, smo ugotovili, da pride do začetka padanja količine biomase veliko kasneje kot v primeru, ko je edini asimilirni vir dušika in regulator pH-ja amonijev hidroksid, kar je razvidno tudi iz grafa 1. Ohranjanje količine biomase na čim višjem nivoju je izredno pomembno pri uporabi kontinuirne fermentacije za proizvodnjo klavulanske kisline.

Med fermentacijo, ki traja 5 do 6 dni vzdržujemo v fazi rasti biomase koncentracijo vira fosforja med 0.005% in 0.15% (w/v). Tekom celotne fermentacije ohranjamo koncentracijo asimilirnega vira dušika med 0.5% in 15% (w/v), najbolje okoli 1%, koncentracija asimilirnega vir ogljika pa se giblje med 7.5% in 1.5% (w/v). Tako dobimo v fermentacijski juhi koncentracijo klavulanske kisline okoli 3500 mg/l (3500 μg/ml), kar je bistveno več glede na znane in v literaturi opisane postopke.

Smatramo, da smo tako izrazito zvišanje dobitka klavulanske kisline v fermentacijski juhi po mikrobiološkem postopku z Streptomyces sp. P 6621 FERM P2804 dosegli tako z ugodnim izborom seva, kot z najboljšim izborom izhodnega gojišča in vzdrževanjem pH-ja s primerno kombinacijo alkalijskih sredstev ter ohranjanjem koncentracije virov fosforja in dušika med fermentacijo v definiranih območjih.

Kolikor nam je znano v literaturi še ni opisano, da bi lahko z dodajanjem vira fosforja in asimilirnega vira dušika regulirali in vodili proces proizvodnje klavulanske kisline z mikroorganizmi rodu Streptomyces.

Doseganje tako visokega dobitka klavulanske kisline v fermentacijski juhi je bilo resnično presenetljivo in nepričakovano.

Fermentacijsko juho nato obdelamo v skladu s postopkom opisanim v našem slovenskem patentu P 9400107 oziroma ekvivalentni PCT patentni prijavi WO 95/23870, po katerem dobimo kot ciljni željeni produkt kalijev klavulanat velike čistote.

Izum prikazujeta, vendar v ničemer omejujeta naslednja primera, ki ponazarjata fermentativni postopek pridobivanja klavulanske kisline.

PRIMER 1

A) GOJITEV STREPTOMYCES SP. P 6621 FERM P 2804

Selekcija in vzdrževanje seva

S selekcijskimi postopki skrbimo za izoliranje čim bolj produktivnih klonov mikroorganizma Streptomyces sp.P 6621. Le najbolj produktivne kulture se shranjujejo in so namenjene kot izvor za naslednje selekcijske cikle.

Kolonijo Streptomyces sp. P 6621 aseptično prenesemo v sterilen poter z 2 ml sterilne vode in homogeniziramo. Fragmente micelija nato prenesemo v poševno agarsko gojišče in inkubiramo na termostatu pri 25°C do zrelosti, kar traja 10 do 14 dni.

Po 8 do 10 dneh bakterija preraste površino agarja s svojim micelijem sivo zelene barve. S površine poševnega agarja postrgamo spore in aseptično nacepimo vegetativno fazo, ki jo inkubiramo na stresalniku 24 ur pri 250 obr./min. in 25° ± 1°C.

Hkrati, ko nacepljamo vegetativno fazo, homogeno suspenzijo spor iz agarja shranimo v suspenziji posnetega mleka ("skim milk"), ki služi kot zaščitno gojišče za shranjevanje kulture za čas do dveh mesecev.

Po končani vegetativni fazi del kulture aseptično prenesemo v fermentacijsko gojišče in inkubiramo pri enakih pogojih na rotacijskem stresalniku 96 ur. Po končani fermentacijski fazi analiziramo vsebnost klavulanske kisline. Kulture, ki dajejo visoke rezultate predstavljajo vcepke za nacepljanje v fermentor (laboratorijski inokulum).

Ves opisan postopek je voden v aseptičnih pogojih.

Sev shranjujemo na poševnih agarjih in rujevkah pri temperaturi 4°C do 4 tedne, spore v posnetem mleku ("skim milk") do 2 meseca pri temperaturi 4 °C, liofilizate več let pri temperaturi 4°C.

Sestava gojišča za selekcijo seva za nacepljanje v fermentor

Gojišče za poševnike, rujevke in Petrijeve plošče:

Sestava	količina
dekstrin	10 g
KH_2PO_4	1 g
$MgSO_4$. 7 H_2O	1 g
NaCl	1 g
$(NH_4)_2SO_4$	1 g
$CaCO_3$	4 g
elementi v sledovih *	1 ml
agar	20 g
voda (demineralizirana)	do 1000 ml

Vse navedene sestavine, razen agarja, vmešamo v 1000 ml demineralizirane vode. Agar raztopimo v tako pripravljenem gojišču šele na koncu. Pred sterilizacijo uravnamo vrednost pH na 7.00 do 7.40 s 30%-no vodno raztopino NaOH. Za pripravo 1000 ml gojišča uporabimo 2000 ml erlenmajerico, ki jo zamašimo z vatnim zamaškom in papirjem ter elastiko. Tako pripravljeno erlenmajerico steriliziramo v avtoklavu v času 20 min. in pri 121°C.

Gojišče za petrijevke in rujevke je enako kot za poševnike.

Predno razlivamo 15 do 20 ml medija v epruveto dimenzije 25 x 250, gojišče zavremo. Epruvete zamašimo z vatnim zamaškom in steriliziramo v avtoklavu 20 min. pri 121°C. Po sterilizaciji je potrebno gojišče dobro mešati, da se kalcijev karbonat enakomerno porazdeli.

Petrijevke in rujevke pripravimo tako, da jih steriliziramo in nato vanje aseptično razlijemo 30 ml steriliziranega gojišča z gornjo sestavo.

Sestava mineralne raztopine (elementi v sledovih*)

Sestavine	količine
$CaCl_2$. $2H_2O$	10.0 g
$MgCl_2$. 6 H_2O	10.0 g
NaCl	10.0 g
$FeCl_3$. 6 H_2O	3.0 g
$ZnCl_2$	0.5 g
$CuCl_2$ .2 H_2O	0.5 g
$MnSO_4$. H_2O	0.5 g
voda -demineralizirana	do 1000 ml

Vegetativno gojišče za selekcijo

Sestavine	količine
koruzni škrob	10.0 g
sojina moka	20.0 g
KH_2PO_4	0.6 g
estol (Priolube 1435)	5.0 g
vodovodna voda	do 1000 ml

Sestavine med mešanjem vsipamo v vodo in nato tako pripravljenemu gojišču uravnamo vrednost pH na 7.00 s 30%-no vodno raztopino NaOH in ga nato porazdelimo na alikvotne dele s po 50 ml v 300 ml erlenmajerice. Slednje zamašimo z vatnim zamaškom, prekrijemo s papirjem in učvrstimo z elastiko ter tako pripravljene steriliziramo v avtoklavu 20 min. pri temperaturi 121°C.

Fermentacijsko gojišče za selekcijo

Sestavine	Količine
koruzni škrob	9.6 g
sojina moka	38.5 g
KH_2PO_4	1.2 g
estol (Priolube 1435)	23.0 g
glicerol	5.0 g
morfolinopropan sulfonska kislina	12.0 g
elementi v sledovih*	10.0 ml
voda (vodovodna)	do 1000 ml

Sestavine dodamo v vodo med mešanjem in razdelimo v alikvotne dele po 25 ml v 300 ml erlenmajerice, ki jih zamašimo z vatastim zamaškom, prekrijemo s papirjem in učvrstimo z elastiko ter steriliziramo v avtoklavu pri 20 min. pri temperaturi 121°C.

Priprava laboratorijskega inokuluma

Izvorna kultura za pripravo laboratorijskega inokuluma (vcepka) predstavljajo kulture na poševnikih oziroma rujevkah. Izbran poševnik oziroma rujevko pripravimo tako, da vanj aseptično vlijemo 10 ml sterilne vode, postrgamo spore, katere homogeniziramo v sterilnem poterju. Raztopina spor predstavlja laboratorijski inokulum.

VEGETATIVNA FAZA V PROPAGATORJU (predpredfermentor)

Gojišče za propagatorje

Vol. propagatorja=500 l
Vol.gojišča= 350 l

Pripravimo hranljivo gojišče s sledečo sestavo:

Sestavine	količine
koruzni škrob	7.0 kg
sojina moka	7.0 kg
NaH_2PO_4	0.185 kg
estol (priolube 1435)*	0.7 kg
synperonic	0.150 kg
voda (pitna)	do 350 l

* Varianta gojišča vsebuje namesto estola enako množino sojinega olja

V gojišče, ki ga steriliziramo v propagatorju in ohladimo ob uvajanju sterilnega zraka na 28°C, nacepimo laboratorijski inokulum. Vegetativno fazo vodimo 50 do 70 ur pri temperaturi 28 °± 1°C ob mešanju, ob nadtlaku 0.3 bara in uvajanju sterilnega zraka.

Rast kulture spremljamo z analizami pH, PMV%, razbarvanja metilenskega barvila in z mikroskopskim pregledom vzorcev. Ko kultura doseže želene parametre rasti, jo precepimo v predhodno pripravljen predfermentor.

Vzorec gibanja parametrov rasti v propagatorju

PON (h)	pH	PMV%	razbarvanje min.
0	7.20	-	-
4	7.25	10	> 5
10	7.35	8	> 5
16	7.30	10	> 5
22	7.20	16	4
28	7.02	17	2.5
34	6.85	18	0.5
39	6.66	20	0.3
45	6.60	21	0.5
51	6.52	22	1.0
56	6.39	22	1.0
61	6.45	20	1.3

Legenda:
PON=dolžina rasti kulture
pH=pH vrednost vzorca
PMV%=volumski % kulture v vzorcu
razbarvanje=čas razbarvanja metilenskega barvila

VEGETATIVNA FAZA V PREDFERMENTORJU

Gojišče za predfermentorje

Vol.predfermentorja=7500 l
Vol.gojišča=4500 l

Pripravimo hranljivo gojišče s sledečo sestavo:

Sestavine	količine
koruzni škrob	90 kg
sojina moka	90 kg
NaH_2PO_4	2.4 kg
estol (priolube 1435)*	9 kg
synperonic	0.5 kg
voda	do 4500 l

* Varianta gojišča vsebuje namesto estola enako množino sojinega olja

V gojišče, ki smo ga sterilizirali v predfermentorju in ohladili ob uvajanju sterilnega zraka in mešanju na 28°C nacepimo vegetativno fazo propagatorja s pomočjo nadtlaka sterilnega zraka. Zrak steriliziramo skozi filtre z velikostjo por 0.2μm.

Vegetativno fazo vodimo 10 do 20 ur pri temperaturi 28°± 1°C ob uvajanju sterilnega zraka in mešanju, pri nadtlaku 0.3 bara.

Rast spremljamo z analizami pH, PMV%, razbarvanje metilenskega barvila in

z mikroskopskim pregledom vzorcev.

Ko kultura doseže želene parametre rasti, jo precepimo v predhodno pripravljen fermentor.

Vzorec gibanja parametrov rasti v predfermentorju

PON (h)	pH	PMV%	razbarvanje (min.)
0	7.20	-	-
6	7.10	15	>5
12	6.87	20	1.5
16	6.65	22	0.3

Legenda:
PON=dolžina rasti kulture
pH= pH vrednost vzorca
PMV%=volumski % kulture v vzorcu
razbarvanje= čas razbarvanja metilenskega barvila

B) ŠARŽNA FERMENTACIJA STREPTOMYCES SP. P 6621 FERM P 2804 V FERMENTORJU

Gojišče za fermentorje

Vol.fermentorja=90 000 l
Vol.gojišča=60 000 l

V fermentorju pripravimo začetno gojišče s sledečo sestavo:

Sestavine	količina
koruzni škrob	570 kg
sojina moka	2300 kg
NaCl	6 kg
estol (Priolube 1435)*	2380 kg
glicerol	1640 kg
NaH_2PO_4	5 kg
$MgCl_2 . 6 H_2O$	7 kg
$FeCl_3 . 6 H_2O$	1.6 kg
$ZnCl_2$	0.5 kg
$MnSO_4 . H_2O$	0.1 kg
synperonic	25 kg
voda	do 60 m^3

Legenda: - estol je generični naziv za glicerol trioleat; (Priolube 1435 - komercialni naziv firme Unichem G.m.b.h., Nemčija)
- Synperonic (komercialni naziv firme I.C.I.,GB) je protipenilec na osnovi propilenglikola

* Varianta gojišča vsebuje namesto estola enako množino sojinega olja

4700 l vegetativne faze kulture Streptomyces sp. PP 6621 FERM P 2804 iz predfermentorja nacepimo sterilno v 60 000 l začetnega gojišča v 90 000 litrskem fermentorju iz nerjavečega jekla, ki je opremljem z mešalom in dovodi za uvajanje sterilnega zraka preko filtrov z velikostjo por 0.2 μm. Gojišče in vse dovode fermentorja steriliziramo. Nato ob dovajanju sterilnega zraka gojišče ohladimo do temperature 24°C ter ga inokuliramo z vegetativno fazo iz predfermentorja, s čemer začnemo fermentacijo, ki jo vodimo pri temperaturi 24°do 25°C ob mešanju in nadpritisku 0.3 bara ter ob kontroliranju pH medija v območju med 6.8 do 6.9 s 25%-no vodno raztopino amonijaka. Fermentacijo vodimo pri 24°C ob mešanju in dovajanju sterilnega zraka in traja 96 ur, pri čemer dobimo koncentracijo klavulanske kisline v fermentacijski juhi, ki znaša 3580 mg/l.

Med fermentacijo kulturi Streptomyces sp. P 6621 FERM P 2804 v začetno gojišče sterilno dodajamo vir fosforja in asimilirni vir dušika (500 kg sojine

moke na 5000 l vode), 25%-no vodno raztopino amonijaka, pri čemer spremljamo za fermentacijo pomembne parametre.

Vzorec gibanja koncentracije vira fosforja in asimilirnega vira dušika v fermentorju

PON	skupni vir P	skupni vir N
0	0,035	1,73975
8	0,030625	1,692286
16	0,0095	1,331
24	0,005188	0,9785
32	0,004638	0,5527
40	0,003638	0,69945
48	0,000863	0,9128
56	0	0,8475
64	0	0,709
72	0	0,653625
80	0	0,571
88	0	0,47675
96	0	0,53825
104	0	0,7555
112	0	0,77025
120	0	0,673375
128	0	0,78725
136	0	0,734625
144	0	0,8985

Legenda:
PON=ure po nacepitvi
P=koncentracija topnega celokupnega fosforja v vzorcu v (w/v)
N=koncentracija celokupnega vira dušika v vzorcu v (w/v)

Opomba: koncentracija topnega celokupnega fosforja v fermentacijski brozgi pade v 51 uri pod mejo detekcije

V začetnih urah rasti kulture pH raste do skoraj 7.5. V tem času se porabi vir fosforja, nato prične nastajati klavulanska kislina, ki znižuje vrednost pH-

ja. Brez vzdrževanja navedene vrednosti pH medija bi se le-ta znižal na nivo, kjer aktivna substanca ne bi nastajala.

PRIMER 2

PODALJŠANJE FAZE RASTI BIOMASE Z UPORABO AMONIJEVEGA SULFATA KOT ASIMILIRNEGA VIRA DUŠIKA IN NATRIJEVEGA HIDROKSIDA KOT REGULATORJA pH

Pripravili smo dve gojišči v dveh fermentorjih (500 l). Gojišči za fermentacijo smo pripravili na enak način, kot v PRIMERU 1 B, le v da smo uporabili proporcionalno manjše količine sestavin. V prvem fermentorju je potekala fermentacija na način opisan v PRIMERU 1 B, potek fermentacije v drugem fermentorju pa se je od fermentacije opisane v PRIMERU 1 B razlikoval le v tem, da smo kot vir asimilirnega dušika od PON=40 (PON=40 pomeni 40 ur po nacepitvi v fermentor) od do PON=60 dodajali 11%-no raztopino amonijevega sulfata s pretokom 9 ml/min, nivo pH-ja pa smo uravnavali z natrijevim hidroksidom. Po PON=60 asimilirnega vira dušika nismo več dodajali. V obeh primerih smo merili viskoznost fermentacijske brozge, ki je proporcionalna količini biomase.

PON (h)	ferm.1, viskoznost (m Pa•s)	ferm.2, viskoznost (m Pa•s)
0	/	/
8	/	/
26	474	551
44	728	714
62	948	998
80	995	1076
98	936	1226
116	824	863
128	628	873

LEK, tovarna farmacevtskih in kemičnih izdelkov d.d.
dr.Miha Kremser,dipl.ing.

PATENTNI ZAHTEVKI

1. Postopek za pripravo klavulanske kisline in njenih farmacevtsko sprejemljivih soli, označen s tem, da mikroorganizem Streptomyces sp. P 6621 FERM P 2804 gojimo pri temperaturi med 20° do 30°C, najbolje pri 23° do 25°C in pri vrednosti pH medija med 6.5 do 7.5, najbolje pri kontroliranem vzdrževanju vrednosti pH medija med 6.8 do 6.9, v aerobnih pogojih in hranljivem gojišču, ki že od pričetka fermentacije vsebuje vir fosforja, asimilirne vire dušika in ogljika ter mineralne soli; med fermentacijo do pričetka nastajanja klavulanske kisline kot regulator rasti v gojišče dodajamo vir fosforja in pazimo, da vseskozi ohranjamo primerno koncentracijo vira dušika.

2. Postopek po zahtevku 1, označen s tem, da pH vrednost medija vzdržujemo med 6.5 in 7.5.

3. Postopek po zahtevku 1, označen s tem, da med fermentacijo vzdržujemo koncentracijo vira fosforja med 0.00% do 0.15% (w/v).

4. Postopek po zahtevku 1 in 3 označen s tem, da do štiridesete ure poteka fermentacije vzdržujemo koncentracijo vira fosforja med 0.005% do 0.05% (w/v)

5. Postopek po zahtevku 1, 3 in 4, označen s tem, da med fermentacijo vzdržujemo koncentracijo asimilirnega vira dušika med 0.5% do 15 % (w/v).

6. Postopek po zahtevku 1, 3, 4 in 5, označen s tem, da je začetna koncentracija asimilirnega vira ogljika višja od 5% (w/v).

7. Postopek po zahtevkih 1, 3 in 4 označen s tem, da kot vir fosforja dodajamo natrijev oziroma kalijev fosfat, ali natrijev oziroma kalijev

dihidrogen fosfat, ali dinatrijev oziroma dikalijev hidrogen fosfat.

8. Postopek po zahtevkih 1 in 5, označen s tem, da kot vir dušika dodajamo sojino moko oziroma derivate pridobljene iz sojine moke; oziroma dodajamo ostale vrste rastlinske moke, kot moko iz bombažnih semen.

9. Postopek po zahtevkih 1, 5 in 7, označen s tem, da kot vir dušika v času vegetativnega dela fermentacije dodajamo sojino moko oziroma derivate pridobljene iz sojine moke; oziroma dodajamo ostale vrste rastlinske moke, kot moko iz bombažnih semen.

10. Postopek po zahtevkih 1 in 5, označen s tem, da kot vir dušika dodajamo amonijev hidroksid.

11. Postopek po zahtevkih 1 in 5, označen s tem, da kot vir dušika dodajamo amonijevo sol, kot amonijev sulfat.

12. Postopek po zahtevkih od 1 do 10, označen s tem, da je način fermentacije šaržna fermentacija ali dohranjevalna fermentacija ali kontinuirna fermentacija.

13. Postopek po zahtevku 1, označen s tem, da je način fermentacije kontinuirna fermentacija in da se kot asimilirni vir dušika tekom fermentacije dodaja amonijev sulfat.

14. Postopek za pripravo klavulanske kisline, označen s tem, da postopek obsega stopnje:

a) selekcija seva in izbor gojišča
b) inokuliranje gojišča z mikroorganizmom Streptomyces sp. P 6621 FERM P 2804
c) fermentacijo gojišča z mikroorganizmom v prisotnosti vira fosforja, asimilirnih virov ogljika in dušika ter mineralnih soli in dodajanje vira fosforja

in asimilirnih virov dušika v gojišče med fermentacijo

d) dobimo fermentacijsko juho, ki vsebuje visoko koncentracijo klavulanske kisline.

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IZVLEČEK

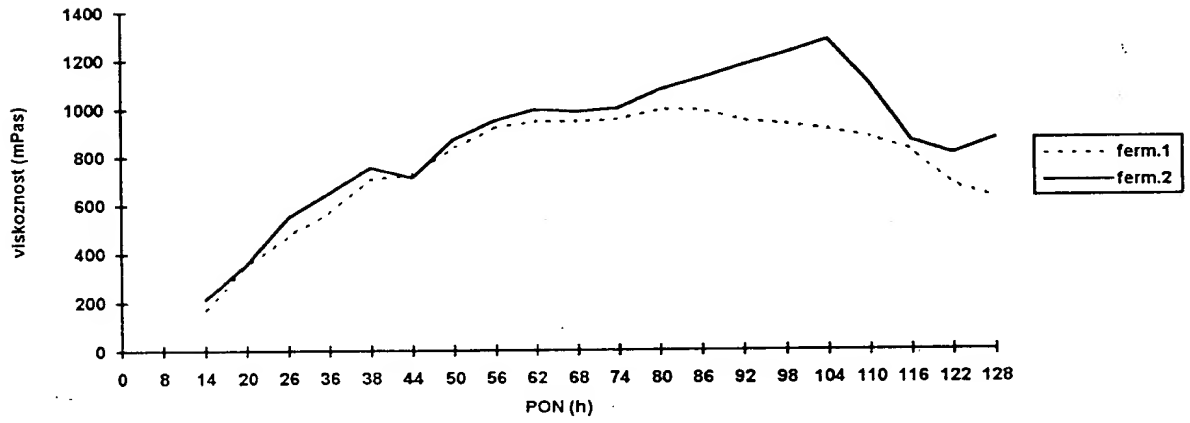
Opisan je nov in izboljšan postopek za pripravo klavulanske kisline in njenih farmacevtsko sprejemljivih soli, po katerem dobimo bistveno zvišan dobitek želene spojine v fermentacijski juhi, po katerem mikroorganizem Streptomyces sp. P 6621 FERM P 2804 gojimo pri temperaturi med 20° do 30°C, najbolje pri 23° do 25°C in pri vrednosti pH medija med 6.5 do 7.5, najbolje pri kontroliranem vzdrževanju vrednosti pH medija med 6.8 do 6.9 z vodno raztopino alkalijskega sredstva, v aerobnih pogojih in hranljivem gojišču, ki že od pričetka fermentacije vsebuje asimilirne vire dušika in ogljika, vir fosforja in mineralne soli ter med fermentacijo do pričetka nastajanja klavulanske kisline kot regulator rasti v gojišče dodajamo vir fosforja in pazimo, da vseskozi ohranjamo primerno koncentracijo vira dušika.

Začetna koncentracija asimilirnega vira ogljika je višja od 5% (w/v), med fermentacijo pa do pričetka nastajanja klavulanske kisline vzdržujemo koncentracijo vira fosforja med 0.00% do 0.15% (w/v).

Dobitki klavulanske kisline v fermentacijski juhi so izredno visoki in znašajo okoli 3500 mg/l.

Graf 1

Primerjava poteka standardne fermentacije (ferm.1) s potekom fermentacije (ferm.2), kjer kot asimilirni vir dušika dodajamo amonijev sulfat in kot regulator pH-ja natrijev hidroksid



Opomba: viskoznost je proporcionalna količini biomase

LEK Pharmaceutical and Chemical Company d.d.

New and improved fermentation process for preparation of clavulanic acid and its salts

Technical Field
(MPK C; 12 P 17/18)

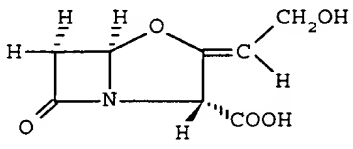
The present invention belongs into the field of pharmaceutical industry and relates to a new and improved process for obtaining clavulanic acid and its salts by fermentation of microorganism *Streptomyces sp. P 6621 FERM P 2804*.

Technical Problem

There exists a constant need for new and improved microbiological process for preparation of clavulanic acid and an additional transformation of it into alkali salt, such as potassium clavulanate, by which the desired compound is obtained in a high yield in a fermentation broth obtained by fermentation of clavulanic acid-producing microorganisms.

Prior Art

Clavulanic acid is the common name for (2R,5R,Z)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]heptane-2-carboxylic acid of the following formula:



Alkali metal salts and esters thereof are active as inhibitors of β-lactamases produced by some Gram positive as well as Gram negative microorganisms. In addition to the action of inhibiting β-lactamases, clavulanic acid and alkali metal salts thereof also have a synergistic action in combination with β-lactam antibiotics of penicillin and cephalosporin series. Therefore clavulanic acid and salts thereof are used in galenic preparations to prevent the deactivation of β-lactam antibiotics. Commercial preparations contain more stable potassium salt of clavulanic acid (the acid alone is rather unstable) in combination with amoxicillin trihydrate, salts thereof or in combination with other antibiotics.

Clavulanic acid is prepared by fermentation of a clavulanic acid-producing microorganisms such as various microorganisms belonging

to different *Streptomyces* strains such as. *S. clavuligerus NRRL 3585*, *S. jumoninensis NRRL 5741*, *S. katsurahumanus IFO 13716* and *Streptomyces sp.P 6621 FERM P 2804*.

Use of *S.clavuligerus NRRL 3585* in a process for preparation of clavulanic acid by fermentation was described in the patent GB 1,508,977, where as the most suitable fermentation medium was characterised medium that contained between 0.1% and 10% of organic nitrogen source, such as yeast hydrolysate, beef and fish extracts, seed's proteins, corn-steep liquor and other hydrolysates (in case of chemically defined medium urea, valine, asparagine, glutamic acid, proline, and phenylalanine were used as nitrogen sources), between 0.1% and 5% of organic carbon source (starch hydrolysate, dextrin, sucrose, lactose, glycerol and esters thereof, vegetable oils and animal fats). Combination of soybean flour, dried breweries solubles and dextrin was shown as especially suitable and cheap medium. Some inorganic salts, such as sodium, potassium, magnesium, iron and zinc chloride, sodium, magnesium and iron sulphate, sodium and potassium salts of phosphoric acid, could be added to chemically defined medium. Salts of manganese, nickel, cobalt, vitamins and antifoam agents for the foaming control could be also added to the fermentation medium. Result of fermentation is a fermentation broth rich with clavulanic acid and its salts.

The aqueous culture broth obtained after fermentation may be purified and concentrated according to conventional process comprising e.g. filtration and chromatographic purification as illustrated in GB 1,508,977 prior to the extraction of the aqueous solution with the organic solvent to obtain a solution of impure clavulanic acid in an organic solvent. GB 1,508,977 teaches, *inter alia*, that salts of clavulanic acid may be obtained by the adsorption of the clavulanate anion in the filtered broth onto an anionic exchange resin and are eluted therefrom with an electrolyte, the solution formed is desalted and then the solvent is removed. This process may be used to achieve acceptable yields of the desired substance, yet it requires sophisticated purification by chromatographic methods and use of resin columns demands

important investments, which limits production operations on a large scale.

Great novelty and improvement, that enabled production operations on a large scale, is illustrated in our Slovenian patent P-9400107 or in equivalent PCT application WO 95/23870, where the method for isolation of clavulanic acid or its pharmaceutically acceptable alkali salts, such as potassium clavulanate, based on removing of mycelium and other suspended particles from the fermentation broth by microfiltration and ultrafiltration. The fermentation broth obtained after micro- and ultrafiltration is concentrated by reverse osmosis and then it can be extracted with a water-immiscible organic solvent that, at the end of the process, gives a desired compound of high purity. By that method most of conventional time-consuming procedures and chromatographic purification of product could be left out.

It is known from the literature, that optimisation of the conditions in the fermenter is necessary for a successful fermentation. Batch fermentation is a dynamic process, where the environment is changing continuously and the microbial cells are all the time subjected to the changes of the environment. Changes in the environment of the cell express themselves as great changes in the cell's physiological condition and living all the time under stress condition cells are prevented to grow optimally and to produce desired metabolites (Yung T.B. et al. Ferm.Technol. 1972, Today 163).

Patent GB 1,543,563 describes a modified fermentation process for preparation of clavulanic acid by strain *S.clavuligerus NRRL 3585*, where the pH value of the medium is kept between 6.3 and 6.7, by which the higher yield of desired compound is achieved.

Besides the physical parameter such as pH, temperature and the content of oxygen, the content of different mineral sources and assimilable sources of nitrogen and carbon can be controlled and kept at the desired level. That is so called feed-batch fermentation and is also well-known from the literature. Use of feed-batch fermentation

for the production of antibiotics is described by Crueger W. et. al., Biotechnology 1984, p. 197-237.

Control and maintenance of the desired level of assimilable source of nitrogen and carbon in the fermentation broth is well illustrated by Lee J.S. et. al., Kor.Jour.Microbiol. 1978, Vol. 15, No. 1, p. 21-29, where the improvement of the fermentation process for preparation of penicillin by control of addition of the assimilable nitrogen and carbon sources as needed by the microorganisms in the fermenter is described.

The article by Lilley G. et. al., J.Chem.Tech.Biotechnol. 1981, Vol. 31, p. 127-134 illustrates that the production of antibiotics by *Streptomyces* species can be controlled by changing the concentration of assimilable nitrogen and carbon sources and a source of phosphorus. For example, the production of thienamycin in the fermenter does no start before the concentration of phosphorus is close to null.

Adding and control of the concentration of an assimilable carbon source in the process for preparation of clavulanic acid has been first disclosed in patent EP-B-0 182 522, where the method of preparing clavulanic acid by the batch fermentation of microorganism *S. clavuligerus NRRL 3585* is described. An important improvement of the process has been achieved by addition of a carbon source such as glycerol to the fermentation medium in the course of the process either continually or intermittently, whereat it is very important that the carbon level is maintained in a sufficiently low concentration, under 0.5% (w/v) and by no means to exceed 2%. The Examples illustrate that the essential improvement of the increased yield of clavulanic acid was observed when the carbon source, such as glycerol, at the beginning of the fermentation was not present and was added in the course of the process either continually or intermittently during fermentation. It is stated that the concentration of clavulanic acid in the fermentation broth after 160 hours was about 1400 μg/ml, this being a noticeable improvement over previous processes.

It is evident from the article Butterworth D., Drugs Pharm.Sci., 1984, Vol. 22 (Biotechnol. Ind. Antibiot.) p. 225-235, where an overview of fermentation processes for preparation of clavulanic acid by *Streptomyces* is made, that all today known processes for preparation of clavulanic acid by *S. clavuligerus* described in the patent literature base on pilot plants and that processes for preparation of clavulanic acid by other *Streptomyces* are limited to a smaller or laboratory scale. Fermentation process for preparation of clavulanic acid described in patent EP-B-0 182 552 is also limited to a pilot plant scale procedures and the literature published before this patent application has not described fermentation of clavulanic acid by *Streptomyces* species on the industrial scale ("large-scale operation").

Technical Solution

This invention is based on the problem how to increase the yield or concentration of clavulanic acid in the fermentation broth obtained by fermentation of microorganism *Streptomyces sp. PP 6621 FERM P 2804*.

This aim has been achieved by cultivation of clavulanic acid producing microorganism *Streptomyces sp. PP 6621 FERM P 2804*, which is described in JP Kokkai Application No.79-72 085 (Early-Disclosure No.80-162993), in the temperature range 20° to 30°C, preferably between 23° and 25°C, and at a pH of the medium between 6.5 and 7.5, preferably by controlled maintaining of the pH value between 6.8 and 6.9 with an aqueous solution of alkali agent, under aerobic condition and in the nutrient medium that contains the source of carbon, the source of nitrogen and mineral salts.

The term cultivation means the deliberate aerobic growth of a clavulanic acid-producing organism in the presence of assimilable sources of carbon, nitrogen and mineral salts. Such aerobic growth may take place in a medium in which the nutrients are dissolved or suspended. The cultivation may take place on an aerobic surface or by submerged culture. The nutritive medium may be composed of complex nutrients or may be chemically defined.

The nutrient media which may be used for the cultivation of *Streptomyces sp. PP 6621 FERM P 2804* may contain from 2 to 15% a complex organic nitrogen source such as yeast extract, meat and fish extracts, vegetable protein such as soybean flour or protein hydrolysates like peptones. Alternatively chemically defined sources of nitrogen may be used such as different amino acids.

As a carbon source, included in the nutrient medium in the concentration range from 1.5 to 7.5%, any assimilable carbon source may be used, preferably those with the chemically defined structure. Starch or starch hydrolysates such as dextrin, sucrose,

lactose, maltose and other sugars or glycerol or glycerol esters such as glycerol trioleate (Estol), vegetable oils such as cotton seed oil may be used as a carbon source.
The addition of an antifoam agent such as Symperonic (supplied by I.C.I., UK) may be necessary to control foaming of certain media in fermenters.

Mineral salts such as $MgCl_2$, $FeCl_3$, $ZnCl_2$, $CuCl_2$, $MnSO_4$, $FeSO_4$, Na_2SO_4 and sodium or potassium salts of phosphoric acid such as NaH_2PO_4 in the concentration around 0.05% may be added to the media described above.
Some trace elements such as cobalt may also be included.

Streptomyces sp. PP 6621 FERM P 2804 may be cultivated in the above described media in Erlenmeyer flasks aerated by shaking on a rotary shaker or in baffled stainless steel fermenters equipped with stirring and sterile air supply devices. Working volume of the fermenter is between 25% to 85% of the total volume, fermentation may be batch or continuous.

Peak yields of clavulanic acid are obtained within 6 days of fermentation.

According to the present invention, where batch fermentation of *Streptomyces sp. PP 6621 FERM P 2804* is described, first the most appropriate clone of the microorganism should be selected. After that a laboratory inoculum is prepared that is a high yielding culture on agar slopes or in Roux bottles.

For fermentation of the microorganism the laboratory inoculum is transferred into the pre-seed tank containing the previously sterilised medium. In this stage the medium has growth-producing properties, is vegetative and its purpose is to increase the number of microorganisms and not to produce clavulanic acid. After a desired growth stage of the culture is achieved (18 to 25% decolorization in 0.5 to 1.5 min) (percentage of decolorization is a measure for culture viability), it is transferred into the seed fermenter with the previously

sterilised medium. After a desired growth stage of the culture in the seed fermenter is achieved, it is transferred into the fermenter with the previously sterilised medium where the microbiological process for preparation of clavulanic acid takes place.

Sources of nitrogen and carbon, and mineral salts such as described above used for the composition of the medium, the procedure of growth and microbiological preparation of clavulanic acid are shown in the Example.

It is evident from the patent EP-B-0 182 522, that the portion of carbon in the medium during inoculation is between 0 and 2% (w/v) (weight/volume), preferably between 0 and 0.5% (w/v). It is illustrated in examples 1-3, where the best results are achieved if no carbon source is present in the starting medium. According to the Example 1, a high yield or concentration of clavulanic acid of about 1400 μg/ml in the fermentation broth after 160 hours was achieved.

In contrast to the microbiological process for preparation of clavulanic acid by fermentation of *S.clavuligerus* as it is described in the patent EP-B-0 182 522, where a significant improvement was achieved if to the starting medium devoid of a carbon sourse and the source of carbon was added during the course of fermentation either continually or intermittently, whereat it was very important that the carbon level was maintained under 0.5% (w/v) or lower and should not exceed 2%, we have surprisingly found that clavulanic acid was produced in the significantly higher concentration in the aqueous fermentation broth by fermentation of microorganism *Streptomyces sp. PP 6621 FERM P 2804* if assimilable sources of carbon and nitrogen and a source of phosphorus were present in the starting fermentation medium and additional amounts of the phosphorus source and the assimilable source of nitrogen were added in the course of fermentation.

According to the aim of this invention we have found that absence of the carbon source in the starting medium, as it is stated in EP-B-0 182 522, is not important and the proportion of a carbon source such

as glycerol, glycerol trioleate or corn starch in the starting medium may be higher than 5% (w/v). For achieving high yields of clavulanic acid of key importance is to have enough phosphorus, always higher than 0.02% (w/v) and lower than 0.15% (w/v). In continuing the fermentation, but not in excess of 40 hours of fermentation, the concentration of phosphorus should be maintained between 0.15% and 0.005% (w/v) by addition of the phosphorus source, thereafter the concentration should decrease to approximately 0% (w/v). This procedure is important for regulation of the biomass growth and to repress premature production of clavulanic acid. (When the production of secondary metabolites starts, the production of alive biomass is slower.)

According to the aim of this invention, further we have found that the addition of ammonium hydroxide to the fermentation broth throughout the fermentation process as the sole source of assimilable nitrogen and at the same time as the pH regulator did not have positive effect on the course of fermentation. Too high concentration of ammonium means poison for microbial cells, too low pH means less effective production of clavulanic acid, and to avoid this at the beginning of fermentation soybean flour as an assimilable source of nitrogen was added, and further in the course of fermentation ammonium hydroxide as a source of nitrogen was added.

When ammonium sulphate instead of ammonium hydroxide as an assimilable source of nitrogen and sodium hydroxide as pH regulator were used, a decrease of the biomass amount started much later than in case when ammonium hydroxide was the only assimilable source of nitrogen and pH regulator, as illustrated in Figure 1. Maintenance of the amount of the biomass at the highest possible level is particularly important if the continuous fermentation process is used for clavulanic acid production.

In the course of fermentation, lasting from 5 to 6 days, a phosphorus source at the level between 0.005% and 0.15% (w/v) should be maintained. Throughout the fermentation the concentration of an assimilable nitrogen source should be maintained between 0.5% and

15% (w/v), preferably 1% and the concentration of an assimilable nitrogen source in the range between 7.5% and 1.5% (w/v). The above described nutrient conditions yield the concentration of clavulanic acid in the fermentation broth of about 3500 mg/L (3500 μ g/ml), which is a significantly higher yield than the yield reached by any today known and in the literature described process.

Our opinion is that this significant increase in the yield of clavulanic acid in the fermentation broth following the microbiological process with *Streptomyces sp. PP 6621 FERM P 2804* has been achieved by the adequate selection of the production strain, optimal selection of the initial fermentation medium, maintenance of pH with anappropriate combination of alkali compounds and by maintaining the concentration of the phosphorus and nitrogen sources within the defined ranges during the fermentation process.

To our knowledge, the process of clavulanic acid production by fermentation of Streptomyces sp. where it is maintained and regulated by adding a phosphorus source and an assimilable source of nitrogen has not yet been described in the literature.

Achieving of such a high yield of clavulanic acid in the fermentation broth has really been surprising and totally unexpected.

The fermentation broth obtained by the above described procedure is then treated in a manner known from our patent application P-9400107 or equivalent PCT Application WO 95/23870 by which potassium clavulanate of high purity as the final product is produced.

This invention is illustrated but not in any way limited by the following examples of the fermentation process for clavulanic acid production..

EXAMPLE 1

A) CULTIVATION OF STREPTOMYCES SP. P 6621 FERM P 2804

Selection and maintenance of the strain

The most productive clones of microorganism *Streptomyces sp. PP 6621 FERM P 2804* were obtained by the selection methods. Only the most productive cultures of the microorganism were stored and further used as the source of microorganism for new selection cycles.

Colony of *Streptomyces sp. PP 6621 FERM P 2804* was aseptically transferred into a sterile potter with 2 ml of sterile water and homogenised. Fragments of mycelium were then transferred to agar slopes and incubated to maturity (for 10 to 14 days) in the thermostat at 25°C.

After 8 to 10 days growth of bacterium grey-green mycelium on the surface of agar slopes developed. The spores were then scraped off from the surface of agar slopes, aseptically inoculated in the seed vegetative medium and incubated at 25°±1°C on a shaker at 250 rpm for 24 hours.

The homogeneous suspension of the spores from agar slopes may be stored in skim milk, used as a protective medium for a period not exceeding 2 months.

After completed vegetative stage, the portion of the culture was aseptically transferred to the fermentation medium and incubated on a rotary shaker for 96 hour. After completed fermentation stage, the content of clavulanic acid was assayed. The cultures giving the highest yields were used as the inoculum in the fermenter (laboratory inoculum).

The whole procedure took place under aseptic conditions.

The strain may be stored on agar slopes at temperature 4°C maximum for 4 weeks, in skim milk under the same conditions for 2 months; the lyophilised strains may be stored at 4°C for several years.

Composition of a medium for selection of strain for inoculation in a fermenter

Medium for agar slopes, Roux bottles and petri plates

Composition	Amount
Dextrin	10 g
KH_2PO_4	1 g
$MgSO_4 . 7 H_2O$	1 g
NaCl	1 g
$(NH_4)_2SO_4$	1 g
$CaCO_3$	4 g
Trace elements *	1 ml
Agar	20 g
Water (demineralised)	to 1000 ml

The above ingredients, with the exception of agar, were mixed in 1000 ml of demineralised water; the agar was dissolved in this medium at the end. Prior to sterilisation the pH was adjusted to 7.00 – 7.40 with 30% aqueous solution ofNaOH. A 2000-ml Erlenmeyer flask, closed with cotton plug and rubber-fixed paper cap, sterilised in an autoclave at 121°C for 20 minutes, was used for preparation of 1000 ml of medium.

The medium for petri plates and Roux bottles was the same as for agar slopes.

The medium was brought to boiling before an aliquot of 15 to 20 ml of the medium was poured into test tubes, size 25 x 250. The

test tubes were closed with cotton plugs and sterilised in an autoclave at 121°C for 20 minutes. After sterilisation the medium was thoroughly stirred to allow even distribution of calcium carbonate.

Petri plates and Roux bottles were sterilised and 30 ml of the sterile medium of the above composition was aseptically transferred into petri dishes and Roux bottles.

Trace elements*

Composition	Amounts
$CaCl_2 . 2H_2O$	10.0 g
$MgCl_2 . 6 H_2O$	10.0 g
NaCl	10.0 g
$FeCl_3 . 6 H_2O$	3.0 g
$ZnCl_2$	0.5 g
$CuCl_2 .2 H_2O$	0.5 g
$MnSO_4 . H_2O$	0.5 g
Water demineralised	to 1000 ml

Vegetative medium for selection

Composition	Amounts
Corn starch	10.0 g
Soybean flour	20.0 g
KH_2PO_4	0.6 g
Estol (Priolube 1435)	5.0 g
Tap water	to 1000 ml

The ingredients were added to the water while stirring, the pH of the medium, prepared in this manner, the pH was adjusted to 7.00

with 30% aqueous solution of NaOH. The medium in 50-ml aliquots was transferred into 300-ml Erlenmeyer flasks. The flasks were closed with cotton plugs and rubber-fixed paper caps and sterilised in an autoclave at 121°C for 20 minutes.

Fermentative medium for selection

Composition	Amounts
Corn starch	9.6 g
Soybean flour	38.5 g
KH_2PO_4	1.2 g
Estol (Priolube 1435)	23.0 g
Glycerol	5.0 g
Morpholinopropane sulphonic acid	12.0 g
Trace elements*	10.0 ml
Tap water	to 1000 ml

The ingredients were added to the water while stirring; and the medium in 25-ml aliquots was transferred into 300-ml Erlenmeyer flasks. The flasks were closed with cotton plugs and rubber-fixed paper caps and sterilized in an autoclave at 121°C for 20 minutes.

Preparation of laboratory inoculum

The origin culture for preparation of laboratory inoculum was cultured on agar slopes and in Roux bottles, respectively. To a chosen agar slope or Roux bottle 10 ml of sterile water was aseptically added; the spores were scraped off and homogenised in a sterile potter. The spore solution represents a laboratory inoculum.

VEGETATIVE PHASE IN A PRE-SEED TANK

Medium for a pre-seed tank

Volume of pre-seed tank = 500 L
Volume of medium = 350 L

The medium of the following composition was prepared:

Composition		Amounts
Corn starch		7.0 kg
Soybean flour		7.0 kg
NaH_2PO_4		0.185 kg
Estol (Priolube 1435)*		0.7 kg
Synperonic		0.150 kg
Tap water	to	350 L

* Soybean oil can be used instead of estol.

The medium, sterilised in a pre-seed tank and cooled by aerated sterile air to 28°C, was inoculated with the laboratory inoculum. The vegetative phase lasted for 50 to 70 hours at temperature 28°C°±1°C while stirring, at overpressure 0.3 Bar, and with sterile air supplied.

Growth of the culture was monitored by analysis of pH, PMV%, decolorization of methylene dye and by microscopic examination of the samples. On achieving the desired growth parameters, it was inoculated in a pre-seed tank, previously prepared for this purpose.

Parameters of growth in a pre-seed tank

PON (h)	pH	PMV%	Decolorization (min)
0	7.20	–	–
4	7.25	10	> 5
10	7.35	8	> 5
16	7.30	10	> 5
22	7.20	16	4
28	7.02	17	2.5
34	6.85	18	0.5
39	6.66	20	0.3
45	6.60	21	0.5
51	6.52	22	1.0
56	6.39	22	1.0
61	6.45	20	1.3

Legend:
PON = time of culture growth
pH = pH value of sample
PMV% = volume % of culture in sample
decolorization = time necessary for decolorization of methylene dye

VEGETATIVE PHASE IN A SEED FERMENTER

Medium for a seed fermenter

Volume of seed fermenter = 7500 L
Volume of medium = 4500 L

The medium of the following composition was prepared:

Composition	Amount
Corn starch	90 kg
Soybean flour	90 kg
NaH_2PO_4	2.4 kg
Estol (Priolube 1435)*	9 kg
Synperonic	0.5 kg
Water	to 4500 L

* Soybean oil can be used instead of estol.

The medium, sterilised in the pre-seed and cooled while stirring to 28 °C and aerated with sterile air, was inoculated with the vegetative phase from the pre-seed tank by sterile air overpressure. The air was sterilised through the filters, porosity 0.2 μm.

The vegetative phase lasted from 10 to 20 hours at temperature 28°±1 °C, while aerating with sterile air and stirring, at overpressure 0.3 Bar.

Growth of the culture is monitored by analysis of pH, PMV%, decolorization of methylene dye and by microscopic examination of the samples.

On achieving the desired growth parameters, the culture is inoculated in a seed tank, previously prepared for this purpose.

Parameters of growth in a pre-seed tank

PON (h)	pH	PMV%	Decolorization (min)
0	7.20	–	–
6	7.10	15	>5
12	6.87	20	1.5
16	6.65	22	0.3

Legend:
PON = time of culture growth
pH = pH value of sample
PMV% = volume % of culture in sample
decolorization = time necessary for decolorization of methylene dye

B) BATCH FERMENTATION OF STREPTOMYCES SP. P 6621 FERM P 2804 IN FERMENTER

Medium for fermenters

Volume of fermenter = 90 000 L
Volume of medium = 60 000 L

In the fermenter the starting medium of the following composition is prepared:

Composition	Amount
Corn starch	570 kg
Soybean flour	2300 kg
NaCl	6 kg
Estol (Priolube 1435)*	1680 kg
NaH_2PO_4	5 kg
$MgCl_2$. 6 H_2O	7 kg
$FeCl_3$. 6 H_2O	1.6 kg
$ZnCl_2$	0.5 kg
$MnSO_4$. H_2O	0.1 kg
Synperonic	25 kg
Water	to 60 m^3

Legend:
- estol is a generic name for glycerol trioleate; (Priolube 1435 is a registered trade mark of Unichem Gmbh, Germany)
- Synperonic (a registered trade mark of I.C.I., GB) is an antifoam agent based on propylene glycol

* Soybean oil can be used instead of estol.

4700 L of the culture of *Streptomyces sp. PP 6621 FERM P 2804* in the vegetative phase of growth from a pre-seed fermenter was inoculated by a sterile transfer in a sterile starting medium (60 000l) in a 90 000 L stainless steel fermenter equipped with stirring device and sterile air was supplied through the filters of 0.2 μm porosity. A fermentation medium and all inlet-pipes were sterilised. While supplying sterile air, the medium was cooled to . 24°C, and inoculated with a vegetative phase from a pre-seed fermenter whereat the fermentation started at 24°-25°C, while stirring and at overpressure 0.3 Bar, the pH of the medium was maintained in a range 6.8 to 6.9 with 25% aqueous solution of ammonium hydroxide. The fermentation was conducted at 24°C while stirring and aerating with sterile air and lasted for 96 hours. This yielded the concentration of clavulanic acid in the fermentation broth of 3580 mg/L .

In the course of the fermentation to the culture of *Streptomyces sp. PP 6621 FERM P 2804* in the starting medium a phosphorus source and an assimilable source of nitrogen (500 kg of soybean flour in 5000 L of water) and 25% aqueous solution of ammonium hydroxide) were added while monitoring the parameters important for the fermentation process.

Concentration of phosphorus source and assimilable nitrogen source the fermenter

PON	total P source	total N source
0	0.035	1.73975
8	0.030625	1.692286
16	0.0095	1.331
24	0.005188	0.9785
32	0.004638	0.5527
40	0.003638	0.69945
48	0.000863	0.9128
56	0	0.8475
64	0	0.709
72	0	0.653625
80	0	0.571
88	0	0.47675
96	0	0.53825
104	0	0.7555
112	0	0.77025
120	0	0.673375
128	0	0.78725
136	0	0.734625
144	0	0.8985

Legend:

PON = time of culture growth

P = concentration of soluble phosphorus (w/v) in the sample

N = concentration of soluble nitrogen (w/v) in the sample

Remark: After 51-hour fermentation the concentration of total soluble phosphorus in the fermentation broth dropped under the detection limit.

In the first hours of the culture growth, the pH value reached almost 7.5. During that time the phosphorus source was consumed and clavulanic acid was started to be produced, which decreased the pH value. Without maintaining the defined pH value of the medium, pH would decrease to the level at which the production of the active substance would not be possible.

EXAMPLE 2

PROLONGATION OF THE VEGETATIVE PHASE OF FERMENTATION BY USE OF AMMONIUM SULPHATE AS AN ASSIMILABLE SOURCE OF NITROGEN AND SODIUM HYDROXIDE AS A REGULATOR OF pH

Two media in two fermenters (500 L each) were prepared. The fermentation media were prepared in a same manner as in EXAMPLE 1 B except that proportionally smaller amounts of the ingredients were used. In the first fermenter the fermentation was run under the same conditions as those described in Example 1 B, and the fermentation conditions in the second fermenter differed from those described in Example 1 B in the following: as an assimilable source of nitrogen 11% aqueous solution of ammonium sulphate at 9 ml/L was added to the fermentation over the period from PON = 40 (PON = 40 means 40 hours after inoculation in the fermenter) to PON = 60, while maintaining the pH value with sodium hydroxide. After 60 hours of fermentation, addition of assimilable source of nitrogen was stopped.
In both cases, viscosity of the fermentation broth was measured which was proportional to the amount of biomass.

PON (h)	ferm.1, viscosity (m Pa•s)	ferm.2, viscosity (m Pa•s)
0	/	/
8	/	/
26	474	551
44	728	714
62	948	998
80	995	1076
98	936	1226
116	824	863
128	628	873

CLAIMS

What we claim is:

1. A process for preparation of clavulanic acid and pharmaceutically acceptable salts thereof, characterised in that microorganism *Streptomyces sp. P 6621 FERM P 2804* is cultivated at temperature between 20° to 30°C, preferably at 23°-25°C, at pH between 6.5 to 7.5, preferably if pH of the medium is maintained at 6.8-6.9, under aerobic conditions and in the fermentation medium that contains a phosphorus source, assimilable sources of nitrogen and carbon and mineral salts from the inoculation; during fermentation, until the clavulanic acid is started to be produced, a phosphorus source is added as a growth regulator and throughout the fermentation the concentration of a nitrogen source is kept at the suitable level.

2. A process according to claim 1, characterised in that the pH of the medium is maintained between 6.5 and 7.5.

3. A process according to claim 1, characterised in that the concentration of a phosphorus source is maintained between 0.00% and 0.15% (w/v) during the course of fermentation.

4. A process according to claim 1 and 3, characterised in that the concentration of a phosphorus source is kept between 0.005% and 0.05% (w/v) until 40th hour of fermentation.

5. A process according to claim 1, 3 and 4, characterised in that the concentration of an assimilable source of nitrogen is maintained between 0.5% and 15% (w/v) during the course of fermentation.

6. A process according to claim 1, 3, 4 and 5, characterised in that the starting concentration of an assimilable source of nitrogen is higher than 5% (w/v).

7. A process according to claim 1, 3 and 4, characterised in that sodium or potassium phosphate and sodium or potassium dihydrogen phosphate and disodium or dipotassium hydrogen phosphate, respectively, is added as a source of phosphorus.

8. A process according to claim 1 and 5, characterised in that as a soybean flour or derivatives thereof or some other sorts of vegetable flour such as cotton seed flour is added to the fermentation broth as a source of nitrogen.

9. A process according to claim 1, 5, and 7, characterised in that soybean flour or derivatives thereof or some other sorts of vegetable flour such as cotton seed flour is added to the fermentation broth during the vegetative phase of fermentation as a source of nitrogen.

10. A process according to claim 1 and 5, characterised in that ammonium hydroxide is added to the fermentation broth as a source of nitrogen.

11. Process according to claim 1 and 5, characterised in that ammonium sulphate is added in the fermentation broth as a source of nitrogen.

12. A process according to claim 1 and 10, characterised in that the fermentation is batch or feed-batch or continuous fermentation.

13. A process according to claim 1, characterised in that the fermentation is continuous fermentation and ammonium sulphate is added during the fermentation as an assimilable source of nitrogen.

14. A process for preparation of clavulanic acid, characterised in that the process comprises the following steps:

a) selection of strain and choice of adequate medium
b) inoculation of a medium by microorganism *Streptomyces sp. P 6621 FERM P2804*

c) fermentation of a medium by microorganism in the presence of a phosphorus source, assimilable sources of carbon and nitrogen and mineral salts and addition of a phosphorus source and an assimilable source of nitrogen to the medium during the fermentation

d) obtaining of the fermentation broth that contains a high concentration of clavulanic acid.

ABSTRACT

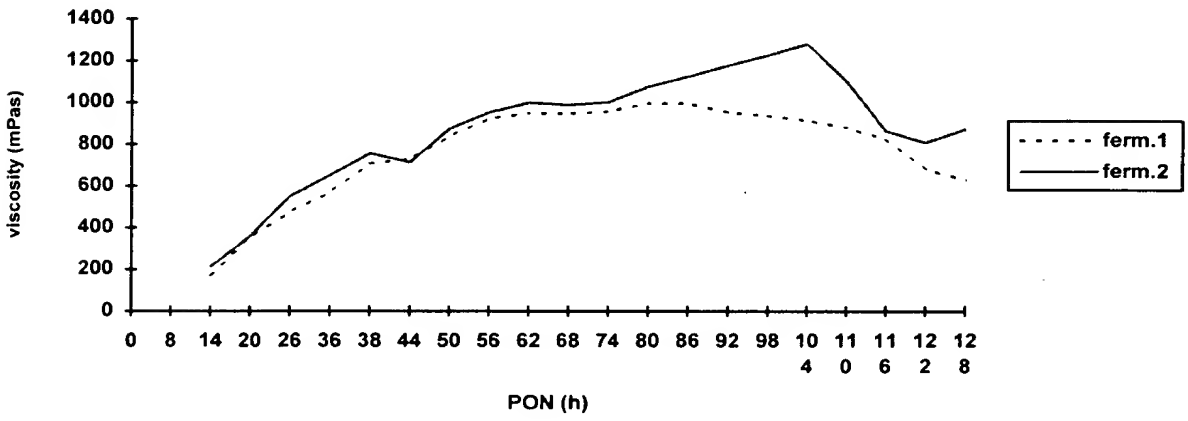
A novel and improved fermentation process for preparation of clavulanic acid and pharmaceutically acceptable salts thereof is described by which a significantly higher yield of the desired compound is obtained in the fermentation broth characterised in that microorganism *Streptomyces sp. P 6621 FERM P 2804* is cultivated at temperature between 20° to 30°C, preferably at 23°-25°C, at pH between 6.5 to 7.5, preferably by controlled maintenance of pH the medium at 6.8-6.9 with an aqueous solution of alkali agent, under aerobic conditions and in the nutrient medium containing assimilable sources of nitrogen and carbon, source of phosphorus and mineral salts from the beginning of the fermentation, and during fermentation until the clavulanic acid is started to be produced a phosphorus source as growth regulator is added taking care to keep the concentration of a nitrogen source at the suitable level throughout the fermentation.

The starting concentration of an assimilable source of nitrogen is higher than 6% (w/v), during fermentation until the clavulanic acid is started to be produced the concentration of a phosphorus source is maintained between 0.00% and 0.15% (w/v).

The yields of clavulanic acid in the fermentation broth are significantly high and amount to about 3500 mg/L.

Figure 1

Comparison of classic fermentation (ferm.1) with fermentation (ferm.2) where ammonium sulphate is the assimilable source of nitrogen and sodium hydroxide is the pH regulator



Remark: Viscosity is proportional to the amount of biomass.

The undersigned Djurdjica Mandrino, permanent court interpreter for the English language, appointed by Decree No. 756-4/91, issued on 11th of February 1991 by the Ministry of Justice and Administration, Republic of Slovenia, hereby declares that this document entirely corresponds to the original Slovenian text.

Ljubljana, 16th may 1995